Identification of *Bacillus subtilis* from Sausage Products and Spices

S. A. PALUMBO, A. I. RIVENBURGH, J. L. SMITH AND J. C. KISSINGER

Eastern Regional Research Center, Philadelphia, Pennsylvania 19118, U.S.A.*

Received 21 May 1974 and accepted 30 September 1974

Bacillus subtilis has been identified as the aerobic spore-former present in several commercial and pilot plant produced sausage products. In our fermented sausages, e.g. Lebanon bolognas, the source of these aerobic spore-formers was the spices, especially black and red pepper, ginger, and allspice. The predominant, if not sole, component of the flora of the spices was *B. subtilis*. On primary isolation from sausage products or from spices, *B. subtilis* formed a volcano-like colony on APT agar. During subculture, even on APT agar, this volcano-like appearance was lost and the typical rough, spreading colony type of *B. subtilis* was observed. Reculturing on sucrose or frankfurter extract agars did not revive the volcano-like colony.

EXAMINATION OF the microflora of food products usually consists of observations on the occurrence, number, and type of food-borne pathogens and/or food spoilage micro-organisms. Components of the indigenous microflora not fitting either of these classes often go unstudied. However, during recent studies of the microbiology of Lebanon bologna (Smith & Palumbo, 1973) and frankfurters (Palumbo, Huhtanen & Smith, 1974), we observed an unusual volcano-like colony (Plate 1) of a Gram positive, catalase-positive spore-forming rod. The unusual form of this colony prompted our characterization of this organism.

We identified it as a member of the genus *Bacillus*. Representatives of the genus *Bacillus* have been found in poultry products (Kraft, 1971), fresh pork sausage (Sulzbacher & McLean, 1951), freshly made liver sausage (Steinke & Foster, 1951), retail ground beef (Rogers & McCleskey, 1957), fresh beef (Halleck, Ball & Stier, 1958; Ayres, 1960; Frazier, 1967), CO_2-stored beef (Mallmann & Zaikowski, 1940), smoked salmon (Lee & Pfeifer, 1973), and dairy products (Witter, 1961; Shehata & Collins, 1971). In most instances, the bacilli were contaminants and did not cause spoilage. However, Shehata, Duran & Collins (1971) found that psychrophilic bacilli can cause spoilage defects in fluid dairy products. Frazier (1967) stated that bacilli are often found as components of the slimes on meat products. In our own work (Palumbo *et al.*, 1974), we observed an increase in the number of aerobic spore-formers during low temperature storage (5°) of one batch of commercial frankfurters. In this case, the Gram positive spore-forming rods constituted the major, if not sole, flora of these frankfurters. Ogilvy (1950) found relatively large numbers of bacilli in commercial frankfurters. Except for the study of Shehata & Collins (1971), none of these

* Agricultural Research Service, U.S. Department of Agriculture.

bacilli has been identified. At this time we cannot assess their importance in terms of food spoilage or food safety. However, we have observed these aerobic spore-formers in a wide variety of sausage products (Lebanon bologna, summer sausage, pepperoni, and various salamis), including those produced in our pilot plant. Thus, we undertook the identification of this unusual volcano-like colony producing bacterium isolated from sausage products and attempted to locate it in the products processed in our pilot plant.

Materials and Methods

The isolates were picked from surface colonies grown on APT (Difco)* agar plates employed to determine the total counts of the various sausage products. The isolates were carried on APT agar slants stored at 5°.

The cultural methods of Smith, Gordon & Clark (1952) were employed to characterize the sausage isolates. To facilitate reference to the various cultures in this study, the sources and strain designations of each isolate and known culture are given in Table 1. The isolates were identified using the classification of Smith *et al.* (1952), Wolf & Barker (1968) and Gordon, Haynes & Pang (1973).

Results

The cultural characteristics of our isolates and the known cultures are given in Tables 2 and 3. All our isolates were Gram positive, catalase-positive spore-forming rods

TABLE 1

Source and strain designation of cultures tested

Source	Strain designation
Known *Bacillus subtilis*	
ARS Culture Collection	NRRL B-543
NRRL, Peoria, Ill.	NRRL B-1650
Isolates from commercial sausage	
All beef summer sausage (Company 1)	B
Summer sausage	C
All beef salami	D
All beef summer sausage (Company 2)	F
Frankfurters	J
Isolates from sausages produced in our pilot plant and from Lebanon spice mixture	
Pork Lebanon bologna	A
Beef Lebanon bologna	E
Frankfurter processed to 155°F (68·3°)	K
Frankfurter processed to 165°F (74°)	M
Lebanon bologna spice mixture	N

* Reference to brand or firm name does not constitute endorsement by the U.S. Department o Agriculture over others of a similar nature not mentioned.

which were identified with the genus *Bacillus* and using the criteria of Smith *et al.* (1952), Wolf & Barker (1968) and Gordon *et al.* (1973), all the isolates were identified as *Bacillus subtilis.*

TABLE 2

Sugar fermentation patterns for unknown isolates and known cultures of* B. subtilis

	Strains†											
Sugar	NRRL B-543	NRRL B-1650	B	C	D	F	J	A	E	K	M	N
Mannitol	A	–	A	A	A	A	A	A	A	A	A	A
Glucose	A	A	A	A	A	A	A	A	A	A	A	A
Lactose	–	–	–	–	–	A	A	–	A	–	–	–
Sucrose	A	A	A	A	A	A	A	A	A	A	A	A
Sorbitol	A	–	–	A	A	A	A	A	A	A	A	A
Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–
Arabinose	A	A	A	A	A	–	A	A	A	A	A	A
Xylose	–	–	–	–	–	–	A	–	–	–	–	A
Salicin	–	A	–	–	A	A	–	–	A	A	A	A

* A,=acid but no gas; +, =no reaction.
† See Table 1 for strain origins and designations.

TABLE 3

Biochemical tests for isolates and known cultures of B. subtilis*

Test	Results for isolates and known cultures	Characteristics of *B. subtilis*
Casein hydrolysis	All strains positive	Positive
Starch hydrolysis	All strains positive	Positive
Gelatin hydrolysis	All strains positive	Positive
Production of acetylmethylcarbinol	All strains positive except M	Positive except one strain
Growth in NaCl-APT broth	Strains NRRL B-1650 and C grew to 7·5%, all the rest grew to 10%	Good growth up to 7%, growth in a few cases to 10 or 12%
Citrate utilization	All strains positive except NRRL B-1650	Positive except for 2 strains
Propionate utilization	All strains negative	Negative
Gas from nitrate anaerobically	All strains negative	Negative
Reduction of nitrate to nitrite	All strains positive	Positive
pH of glucose broth (7 days)	5·5 to 7·0	5·0 to 8·7
Anaerobic growth	Negative	Negative
Black pigment	All strains negative on glucose and tyrosine nutrient agars; strains A, B, and D were positive in glucose broth	Negative except for varieties
Pellicle in nutrient broth	All strains positive	Positive

* See Table 1 for strain origins and designations.

The following characteristics were utilized in identifying our isolates. They had non-swollen sporangia and central, oval to cylindrical spores. Thus we placed them in Group I of the genus *Bacillus* (Smith *et al.*, 1952; Wolf & Barker, 1968; Gordon *et al.*,

1973). Further important characteristics of the isolates were: hydrolysis of starch, reduction of nitrate to nitrite, acid production (but no gas) in glucose–ammonium chloride–salts medium, production of acetylmethylcarbinol, no anaerobic growth, and no gas from nitrate under alkaline anaerobic conditions. Our isolates agreed in these characteristics with known strains of *B. subtilis* (Smith *et al.*, 1952; Breed, Murray & Smith, 1957; Wolf & Barker, 1968; Gordon *et al.*, 1973). Size ranges of cells (0·5 to $1{\cdot}1 \times 1{\cdot}3$ to 3 μm) and of spores (0·5 to $0{\cdot}6 \times 1{\cdot}0$ to 1·5 μm) were within the ranges given for *B. subtilis* (Smith *et al.*, 1952; Breed *et al.*, 1957).

The possibility that our isolates might be *B. subtilis* var. *aterrimus* or *B. subtilis* var. *niger* was investigated by inoculating the isolates on to glucose nutrient agar (GNA) and tyrosine nutrient agar (TNA) (Smith *et al.*, 1952) and examining them periodically for production of black pigment. None was observed. However, strains A, B, and D produced a black pigment just below the surface of the glucose broth used for acetylmethylcarbinol production and in APT broth (Difco). Since GNA and TNA are considered the media of choice for production of the black pigment, strains A, B, and D were not considered to be the varieties *aterrimus* or *niger*.

The specific culture tests of the various isolates are given in Tables 2 and 3. The sugar fermentation patterns for all 13 strains are given in Table 2. In general, there was good agreement between these patterns for the known *B. subtilis* and our isolates. According to Bergey's Manual (Breed *et al.*, 1957), *B. subtilis* should be positive for xylose; however, only strains J and N were positive. There was good agreement between our isolates and the known cultures in the metabolism of the 3 additional sugars: sorbitol, rhamnose, and salicin. One further test was applied to our isolates and the known cultures: production of gas in Jensen's spiced ham broth. All cultures except strains K and M produced gas in Jensen's broth at 37° while all were negative at 25° and 49°.

These bacilli were isolated from sausage products made in our pilot plant and those produced commercially. An attempt to locate the source of these organisms in our own products was made. We examined the beef and pork used in our sausages bacteriologically and found $< 1 \times 10^2$ bacteria/g (the lowest number we could detect). Next, we examined the spice mixtures, sugars, and salt used. The sugars, salt, and the frankfurter spice mixture were low in micro-organisms (< 5/g), while the Lebanon bologna spice mixture (Palumbo, Smith & Ackerman, 1973) contained *c.* 1×10^7 of aerobic spore-formers/g (Table 4). Because of the large quantities of slime formed by these organisms on primary isolation on APT agar and their spreading growth, exact counts were not possible. Examination of the individual spices of the mixture (Palumbo *et al.*, 1973) showed that several contained large numbers of these organisms (Table 4). Their predominant, if not sole, flora appeared to be the volcano-like colonies of *B. subtilis*.

Originally, we identified these organisms from their unusual surface colonies on inverted APT agar plates. We have termed this colony type volcano (see Plate 1); however, after 2 or 3 subcultures, even on APT agar, this form was lost, and subsequent colonies resembled the typical rough, spreading ones of *B. subtilis*. Attempts were made to restore volcano-colony formation by culturing our isolates on frankfurter nutrient agar (Difco nutrient agar + aqueous extract of frankfurter), sucrose nutrient agar (Difco nutrient agar + 2% sucrose) (Owen, 1923), and sucrose APT agar (Difco APT agar + 2% sucrose) (Owen, 1923). None of these media stimulated a

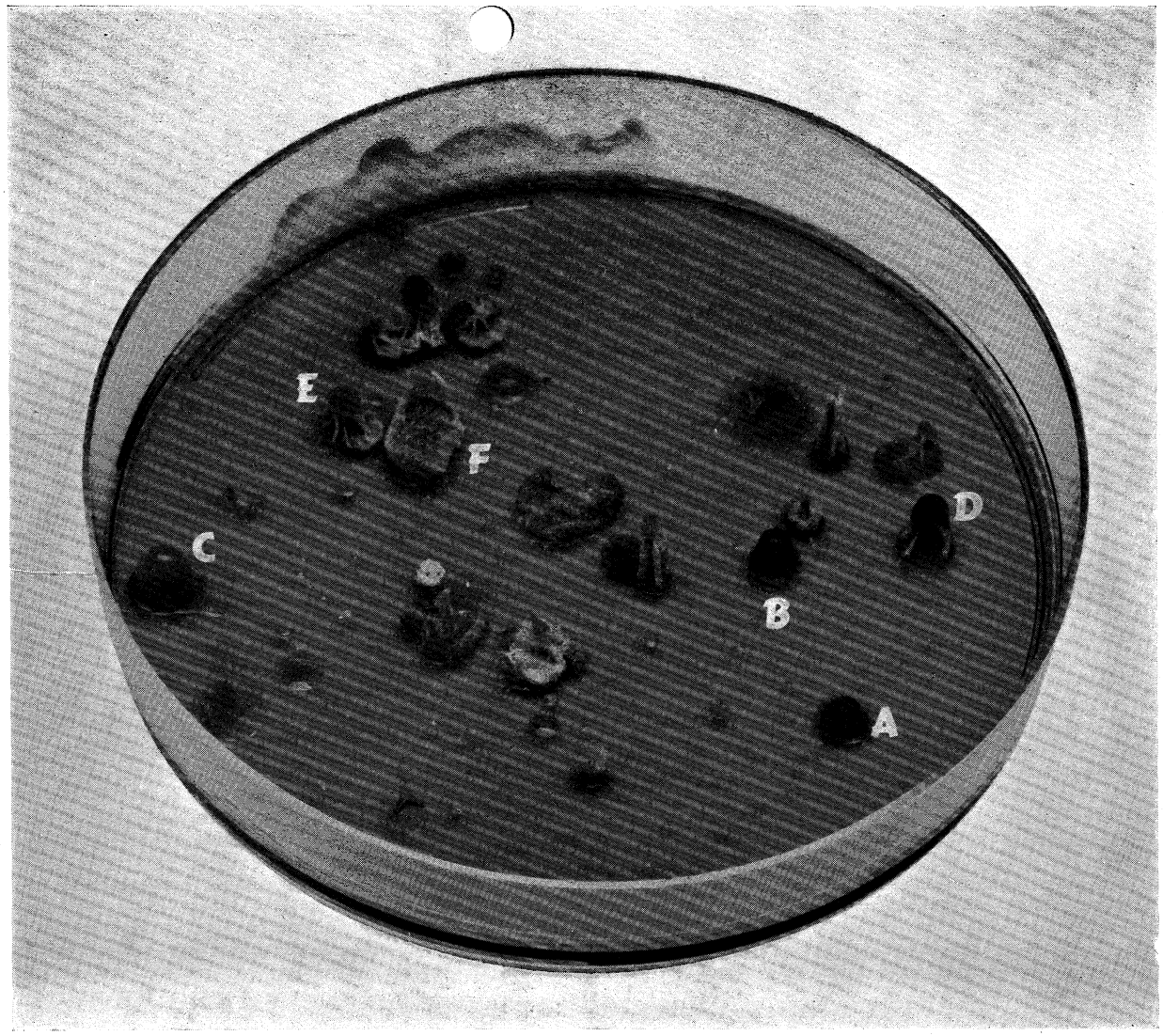

PLATE 1. Photograph of 'volcano' colonies of *B. subtilis* on APT agar; plates incubated inverted for 3 days at 25°. A to F show the successive formation of the 'volcano' colonies. Source of inoculum: summer sausage.

recurrence of this colony type. Formation of volcano colonies is believed to be nutrient dependent because the organism did not form them on primary isolation when tryptic soy agar (Difco) was used.

TABLE 4

*Bacteriological examination of component used to prepare Lebanon bologna**

Spice component	Count/g†
Lebanon bologna spice mixture	1×10^7
Black pepper	2×10^7
Ginger	$1{\cdot}5 \times 10^7$
Allspice	5×10^6
Red pepper	$1{\cdot}6 \times 10^6$
Cinnamon	1×10^5
Nutmeg	1×10^5
Cloves	2×10^3
Mace	1×10^3
Mustard	$< 1 \times 10^3$

* One gram of spice mixture or individual spice was weighed on a sterile weighing dish and added to 99 ml of sterile 0·1% peptone water. Appropriate dilutions of this were surface plated on APT agar and incubated for 2 to 4 days before counting.

† Because of the spreading nature of growth, the counts are approximations. However, they do indicate the general level, especially for the individual spices. In all instances, the only colony type present was the 'volcano colony' of *B. subtilis*.

Discussion

We have identified *B. subtilis* as the Gram-positive, catalase-positive aerobic sporeforming rod present in a wide variety of sausage products. One culture, strain M, could possibly be another species because of its failure to produce acetylmethylcarbinol. Gordon *et al.* (1973) stress production of acetylmethylcarbinol in their stepping-stone key to identifying strains of bacilli. Strain M, however, is identical to the known cultures and the other isolates in all other characteristics. Thus we also included it in the designation of *B. subtilis*. Furthermore, Breed *et al.* (1957) and Gordon *et al.* (1973) have found strains of *B. subtilis* negative for acetylmethylcarbinol production.

The source of these organisms in fermented sausages prepared in our pilot plant appeared to be the spices (Table 4). Spices often contain large numbers of bacteria (Fabian, Krehl & Little, 1939; Yesair & Williams, 1942; Christensen *et al.*, 1967) and moulds which can be introduced into food products. The observation that our Lebanon bologna spice mixture contained *B. subtilis* almost entirely (Table 4) was, however, unusual.

These aerobic sporeformers were recovered from our sausages at levels of *c.* 10^2 to 10^3/g. This represented a decrease of at least one, and probably two, log cycles

from the number added, e.g. the number added by the spice mixture should give a count of 10^4 to 10^5/g. After 24 h fermentation in Lebanon bologna, the number isolated was 10^3/g or less. The small numbers of these organisms which survive the early part of the fermentation continued to survive at least 6 months to 1 year in the sausage stored at 5°.

The activity of *B. subtilis* in these sausages is unknown, but Jensen & Hess (1941*a*) found that members of the *B. subtilis–B. cereus* group are important in canned hams where they produce large quantities of CO_2 from sugar. Our isolates seem to be metabolically inactive in sausage products, although most were able to form gas in Jensen's spiced ham broth. Possibly, the low pH (4·5) of Lebanon bologna deters their growth and CO_2 production. Furthermore, certain spices, such as cloves and cinnamon used to prepare Lebanon bologna, have known inhibitors of *B. subtilis* (Fabian *et al.*, 1939).

In general, the bacilli found in meat products or other foods do not grow at low temperatures (Steinke & Foster, 1951; Rogers & McCleskey, 1957). Halleck, Ball & Stier (1958) noted that the few bacilli they observed were not the normal flora of fresh beef. However, bacilli can cause swells in canned hams (Jensen & Hess, 1941*a*), sours and bone taints in hams (Jensen & Hess, 1941*b*), and various defects in fluid dairy products (Shehata *et al.*, 1971). Previously (Palumbo *et al.*, 1974), we observed an increase of these aerobic sporeformers during low temperature (5°) storage of one batch of commercial frankfurters. There were not enough of these frankfurters to store for the long period required for development of such quality defects as sliminess, off-odour, or off-colour. Examination of a second batch of this brand of frankfurters showed none of these organisms and none developed upon extended storage.

The volcano colonies previously referred to were quite slimy and often could be drawn out into long strands with an inoculating loop. Mucoid forms of an organism named *B. subtilis* are known (Chester, 1903; Owen, 1923). Sucrose appeared to stimulate levan formation by *B. vulgatus* culture similar to *B. mesentericus fuscus* or *B. subtilis* var. *viscosus* described by Chester (1904) to form mucus on potato. However, as indicated, sucrose added to either nutrient or APT agars failed to stimulate volcano colony formation once this characteristic was lost.

Our bacteriological analysis of the individual spices of the Lebanon bologna spice mixture (Table 4) agrees well with the data of Yesair & Williams (1942), who found that mustard, cloves, and mace had relatively low levels of sporeformers. Our finding of an almost exclusive flora of sporeformers in the spices agrees with Jensen, Wood & Jansen (1934) who found an exclusive flora of 5×10^6 hay bacilli (*B. subtilis*)/g of coriander and white pepper, but is in contrast to Christensen *et al.* (1967) who found a wide spectrum of bacterial types during their examination of red and black pepper. They did not give the relative proportions of each type, but they did observe some bacilli.

The public health significance of this apparently widespread occurrence of *B. subtilis* in sausage products is unknown. However, *B. subtilis* has been implicated in a few cases of food poisoning (Bryan, 1969) and future research should be conducted into the conditions in meat products which would permit growth.

References

AYRES, J. C. (1960). Temperature relationships and some other characteristics of the microbial flora developing on refrigerated beef. *Fd Res.* **25**, 1.

BREED, R. S., MURRAY, E. G. D. & SMITH, N. R. (1957). *Bergey's Manual of Determinative Bacteriology*. 7th ed. Baltimore: The Williams and Wilkins Company.
BRYAN, F. L. (1969). Infections due to miscellaneous micro-organisms. In *Food-borne Infections and Intoxications*. Ed. H. Riemann. New York: Academic Press.
CHESTER, F. D. (1904). Observations on an important group of soil bacteria. Organisms related to *Bacillus subtilis*. Delaware Coll. Agri. Expt. Stat. 15th Ann. Rept for 1903. p. 42.
CHRISTENSEN, C. M., FANSE, H. A., NELSON, G. H., BATES, F. & MIROCHA, C. J. (1967). Microflora of black and red pepper. *Appl. Microbiol.* **15**, 622.
FABIAN, F. W., KREHL, C. F. & LITTLE, N. W. (1939). The role of spices in pickled-food spoilage. *Fd Res.* **4**, 269.
FRAZIER, W. C. (1967). *Food Microbiology*. 2nd ed. New York: McGraw Hill Company.
GORDON, R. E., HAYNES, W. C. & PANG, C. H.-N. (1973). The genus *Bacillus*. Agriculture Handbook No. 427. U.S. Department of Agriculture.
HALLECK, F. E., BALL, C. O. & STIER, E. F. (1958). Factors affecting quality of prepackaged meat. IV. Microbiological studies. A. Cultural studies on bacterial flora of fresh meat; classification by genera. *Fd Technol., Champaign* **12**, 197.
JENSEN, L. B. & HESS, W. R. (1941*a*). Fermentation in meat products by the genus *Bacillus*. *Fd Res.* **6**, 75.
JENSEN, L. B. & HESS, W. R. (1941*b*). A study of ham souring. *Fd Res.* **6**, 273.
JENSEN, L. B., WOOD, I. H. & JANSEN, C. E. (1934). Swelling in canned chopped hams. *Ind. Eng. Chem.* **26**, 1118.
KRAFT, A. A. (1971). Microbiology of poultry products. *J. Milk Fd Technol.* **34**, 23.
LEE, J. S. & PFEIFER, D. K. (1973). Aerobic microbial flora of smoked salmon. *J. Milk Fd Technol.* **36**, 143.
MALLMANN, W. L. & ZAIKOWSKI, L. (1940). Effect of CO_2 on growth of meat spoilage organisms at low temperatures. *Natl. Provisioner* Aug. 17, 1940.
OGILVY, W. S. (1950). Storage of meat in carbon dioxide atmospheres at temperatures above freezing. Ph.D. Thesis, Iowa State College.
OWEN, W. L. (1923). A study of the formation of gum levan from sucrose. *J. Bactiol.* **8**, 421.
PALUMBO, S. A., HUHTANEN, C. N. & SMITH, J. L. (1974). Microbiology of the frankfurter process: *Salmonella* and natural aerobic flora. *Appl. Microbiol.* **27**, 724.
PALUMBO, S. A., SMITH, J. L. & ACKERMAN, S. A. (1973). Lebanon bologna. I. Manufacture and processing. *J. Milk Fd Technol.* **36**, 497.
ROGERS, R. E. & MCCLESKEY, C. S. (1957). Bacteriological quality of ground beef in retail markets. *Fd Technol. Champaign* **11**, 318.
SHEHATA, T. E. & COLLINS, E. B. (1971). Isolation and identification of psychrophilic species of *Bacillus* from milk. *Appl. Microbiol.* **21**, 466.
SHEHATA, T. E., DURAN, A. & COLLINS, E. B. (1971). Influence of temperature on the growth of psychrophilic strains of *Bacillus*. *J. Dairy Sci.* **54**, 1579.
SMITH, N. R., GORDON, R. E. & CLARK, F. E. (1952). Aerobic sporeforming bacteria. U.S. Dept. Agri. Monograph No. 16.
SMITH, J. L. & PALUMBO, S. A. (1973). Microbiology of Lebanon bologna. *Appl. Microbiol.* **26**, 489.
STEINKE, P. K. W. & FOSTER, E. M. (1951). Microbial changes in refrigerated liver sausage. *Fd Res.* **16**, 245.
SULZBACHER, W. L. & MCLEAN, R. A. (1951). The bacterial flora of fresh pork sausage. *Fd Technol. Champaign* **5**, 7.
WITTER, L. D. (1961). Psychrophilic bacteria—a review. *J. Dairy Sci.* **44**, 983.
WOLF, J. & BARKER, A. N. (1968). The genus *Bacillus*: Aids to the identification of its species. In *Identification Methods for Microbiologists*. Part B. The Society for Applied Bacteriology—Technical Series No. 2. Eds B. M. Gibbs & D. A. Shapton. London: Academic Press, pp. 93–109.
YESAIR, J. & WILLIAMS, O. B. (1942). Spice contamination and its control. *Fd Res.* **7**, 118.